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Detection Rate and Intratumoral Virus Load of Human Herpesvirus-8 in Immunodeficiency-Related B-Cell Lymphoid Malignancies

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Human herpesvirus-8 (HHV-8), associated with Kaposi's sarcoma, primary effusion lymphoma, and Castleman's disease, has been found in circulating B-cells and might have a causative role in B-cell malignancies associated with immunodeficiency syndromes. We determined the rate of detection and intratumoral virus load of HHV-8 by means of a semiquantitative approach in post-transplant lymphoproliferative diseases (PTLDs), AIDS-related non-Hodgkin's lymphomas (NHLs), including both Burkitt's lymphomas (BLs) and large cell lymphomas (LCLs), as well as in control groups consisting of follicular hyperplasias (FHs) and HIV-negative LCLs. HHV-8 sequences were detected at a similar rate in HIV-negative PTLDs (24%), HIV-negative LCLs (22%) and HIV-negative FHs (17%). The detection rate was significantly higher in HIV-positive BLs (73%), HIV-positive LCLs (67%), and HIV-positive FHs (65%) supporting the view of an epidemiological link between HHV-8 and HIV infections. The viral load was 10^2 genome copies per cell in the single case of primary effusion lymphoma included in the LCL group while it was 10^{-3} copy per cell (median value; range: 10^{-4}–10^{-1}) in all the other HHV-8-positive samples. No significant difference of viral load was found according to HIV status. The virus loads of PTLDs and HIV-positive LCLs were significantly higher than those observed in HIV-positive BLs and FHs, suggesting, to some extent, that the degree of immunodeficiency may influence HHV-8 replication. However, with the exception of the single case of primary effusion lymphoma studied, the low intratumoral load of HHV-8 strongly argues against a direct causative agent of the virus in the occurrence of PTLDs and AIDS-related NHLs. ***J. Med. Virol. 53:277–281, 1997.*** © 1997 Wiley-Liss, Inc.

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INTRODUCTION

HIV-infected patients are at high risk of developing high-grade non-Hodgkin's lymphomas (NHLs). These NHLs are characterized by their histological heterogeneity, including different types (anaplastic large cell, centroblastic, immunoblastic, Burkitt's lymphoma, polymorphic lymphoproliferation) and the high frequency of Burkitt's lymphoma (BL). Most of these lymphomas are related to immunodeficiency; similarly, lymphoid malignancies are observed in post-transplant patients. Epstein-Barr virus is often associated with both AIDS-related and post-transplant lymphoproliferative diseases, but immunodeficiency-related lymphomagenesis is thought to be a multistep process, the molecular basis of which is poorly understood.

A novel herpesvirus, human herpesvirus-8 (HHV-8) also known as Kaposi's sarcoma-associated herpesvirus (KSHV), was discovered recently [Chang et al.,

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1994] and was found to be strongly associated with an AIDS-related neoplasm, Kaposi's sarcoma [Moore and Chang, 1995; Dupin et al., 1995]. HHV-8 infection was also associated with Castleman's disease [Soulier et al., 1995; Gessain et al., 1996] and primary effusion lymphoma [Cesarman et al., 1995; Nador et al., 1996]. B lymphocytes were found to be the main target cells of HHV-8 in vivo [Ambroziak et al., 1995]. Moreover, the determination of the nucleotide sequence of HHV-8 DNA has shown recently the presence of genes encoding homologues to cell proteins that may play a role in cell transformation, including interleukin-6 (IL-6), Bcl-2, and cyclin D homologues [Russo et al., 1996].

These data taken together raised the question of the causative role of HHV-8 in NHLs in patients submitted to long-term immunosuppression, as in the course of HIV infection or following organ transplantation. HHV-8 was found to be strongly associated with body cavity-based B-cell lymphoma [Cesarman et al., 1995], a rare form of acquired immunodeficiency syndrome (AIDS)-associated NHL, which is now designated as primary effusion lymphoma [Nador et al., 1996]. HHV-8 was not detected in any other form (i.e. different from primary effusion lymphoma) of NHL tested, which was confirmed in some further studies [Pastore et al., 1995; Gaidano et al., 1996; Marchioli et al., 1996]. In contrast, other investigators demonstrated the presence of HHV-8 DNA sequences in some NHL samples obtained either from HIV-positive or HIV-negative subjects [Luppi et al., 1996a,b; Otsuki et al., 1996; Bigoni et al., 1996; Corbellino et al., 1996a]. These apparent discrepancies might be due either to major epidemiological differences among the populations tested or to major differences in the sensitivity of the PCR tests used for HHV-8 detection. Moreover, most of the studies reported so far did not mention the HHV-8 load in the specimens tested, which might impair the interpretation of results. This led us to investigate both the detection and the intratumoral virus load of HHV8 by means of a semiquantitative approach in post-transplant lymphoproliferative diseases (PTLDs) and AIDS-related NHLs and in control groups.

MATERIALS AND METHODS

Tumor Specimens, Histology, and Clinical Data

Seventeen cases of PTLD and 53 cases of AIDS-related NHL consisting of 26 Burkitt's lymphomas (BLs) and 27 large cell lymphomas (LCLs), including one primary effusion lymphoma were analyzed. In parallel, we studied 32 cases (20 HIV-positive and 12 HIV-negative) of follicular hyperplasia (FH) and 23 cases of HIV-negative LCL. Cases associated with Kaposi's sarcoma had been excluded in the preliminary analysis. All samples were obtained after surgical biopsy was undertaken for diagnosis. In all cases, a fraction of the sample was snap-frozen in liquid nitrogen and kept at −80°C until used for virological studies. The remaining part of the biopsy was submitted routinely to formalin fixation and paraffin embedding. Conventional histological studies were carried out on paraffin-embedded material using hematoxylin and eosin (H&E) staining and Giemsa's method. All cases of NHL were classified according to the Revised European American classification of Lymphoid neoplasms (REAL) classification. HIV status was determined by a serological approach combining enzyme-linked immunosorbent assay (ELISA) and Western blot assays. Other biological and clinical findings were also analyzed for concordance with histological findings and to exclude Kaposi's sarcoma-associated cases.

Detection and Quantification of HHV-8 by Means of PCR

Samples were coded and tested blindly with respect to histological findings and HIV status. DNA extraction and PCR were carried out essentially as described previously [Corbellino et al., 1996b] with minor modifications. Briefly, a 40-cycle PCR was undertaken starting from 1 μg of biopsy DNA. β-Globin gene amplification was used to check both the absence of major Taq polymerase inhibitors and quantify the number of cell genomes in the sample by end-point dilution PCR [Dupin et al., 1995]. HHV-8 DNA sequences were detected using the $KS330_{233}$ primer pair described previously [Chang et al., 1994]. PCR products were electrophoresed in agarose gels and transferred onto nylon membranes for hybridization with ^{32}P-labeled oligonucleotide probes. β-Globin and HHV-8-specific PCRs were performed in parallel independent assays; each DNA dilution was tested in duplicate experiments. HHV-8 virus load was estimated from PCR results obtained on serial 10-fold dilutions of sample DNA as reported previously [Dupin et al., 1995]. Strict measures were undertaken to avoid intersample carryover: independent DNA extractions, disposable tools, and separate rooms for the different steps of analysis. Negative controls and blank reactions were included in each PCR run.

Statistical Analyses

Two-group comparisons were carried out using Fisher's exact test for categories and Mann-Whitney U unpaired test for viral loads as appropriate. Statistical analysis was done with Statview software (Abacus Concepts, Berkeley, CA).

RESULTS

HHV-8 DNA was detected in 37 of 53 (70%) AIDS-related NHL samples and 50 out of 73 (68%) samples from HIV-positive patients including both AIDS-related NHLs and FHs, as shown in Table I. These rates were significantly higher than those observed in samples from HIV-negative patients (21%; $p < 0.001$, Fisher's exact test). This significant difference between HIV-positive and HIV-negative patients found in cumulative analysis was also observed when comparing PTLDs to any of the HIV-positive sample subgroups, as well as within LCL and FH subgroups (Table I). No significant difference of detection rate was found between HIV-positive BLs, LCLs, and FHs (73%, 67%,

TABLE I. Frequency of HHV-8 Detection in the Samples Studied

Histology	HIV status	No.	No. (%) of HHV-8-positive samples	Significant differences in two-group comparisons (*P* value)*				
PTLD	−	17	4 (24)	x	x	x		
BL	+	26	19 (73)	x				
LCL	+	27	18 (67)		x		x	
	−	23	5 (22)				x	
FH	+	20	13 (65)			x		x
	−	12	2 (17)					x
All samples	NA	125	61 (49)					
				0.002	0.01	0.02	0.002	0.01

*Only significant differences evidenced from all pairwise comparisons are cited. The groups compared are indicated by crosses.
NA, not applicable.

and 65%, respectively) in two-group comparisons. Stratification within the LCL group according to histological subtypes did not permit the demonstration of any significant difference of HHV-8 detection rate between these distinct histological subtypes (not shown). Conversely, no significant difference was observed in two-group comparisons between the distincts subgroups (PTLDs, LCLs, and FHs) of HIV-negative subjects (24%, 22%, and 17%, respectively). These results indicated that the rate of HHV-8 infection was related to HIV status, rather than to histological findings.

The virus load of HHV-8-positive specimens was quantified by end-point dilution as exemplified in Figure 1. The virus load was evaluated according to a semiquantitative method based on the last dilutions, giving a positive signal in duplicate assays for HHV-8 DNA and β-globin gene. One sample had a virus load of 10^2 copies per cell and was found to be the single primary effusion lymphoma case of the study. For all the other samples, the virus load ranged from 10^{-4} up to 10^{-1} copy per cell (Table II) with a median value of 10^{-3}. The virus loads of PTLDs and HIV-positive LCLs (median values: 3×10^{-2} and 10^{-2} copy per cell, respectively) were significantly higher than those of BLs and HIV-positive FHs (median values: 10^{-3} and 10^{-4} copy per cell, respectively). Of interest, two Kaposi's sarcoma-associated cases (one HIV-positive FH and one PTLD, both initially included in our study and subsequently withdrawn in final analysis) exhibited a virus load of 1 copy per cell (not shown). This relatively high virus load emphasized the need for exclusion of Kaposi's sarcoma-associated cases when studying the presence of HHV-8 in B-cell malignancies. Thus, in contrast to HHV-8 detection rate, the virus load appeared to be independent of the presence of HIV infection. Rather the higher virus load in PTLDs and HIV-positive LCLs than in either Bls or FHs suggested, to some extent, that HHV-8 replication was related to the level of immunodeficiency. However, even in the cases of PTLDs and HIV-positive LCLs, viral load was low and viral infection obviously implicated only a minority of the cells present in the tumor.

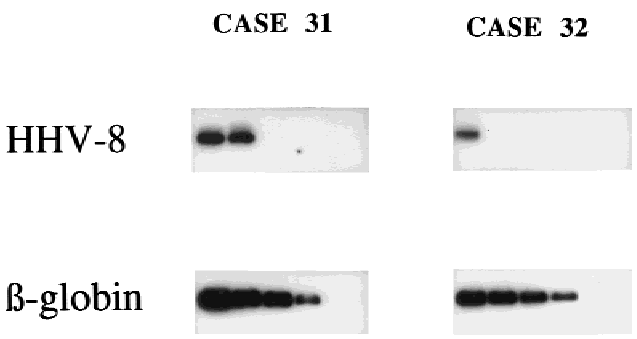

Fig. 1. Quantitation of HHV-8 load in two distinct HHV-8-positive samples. End-point dilution PCR was performed by amplifying six serial 10-fold dilutions of each sample, starting from 1 μg DNA. Each sample was independently amplified with HHV-8 and human β-globin specific primers, as described in Materials and Methods. Each specific PCR assay was done in duplicate. Samples were blindly tested (the two cases depicted here are HIV-positive follicular hyperplasias).

DISCUSSION

In this study, PCR was used for the detection of HHV-8 genome and its quantitation in a large series of B-cell lymphoid malignancies. These lymphoproliferative diseases were diagnosed in immunocompromised subjects consisting of HIV-infected patients and of patients submitted to a continuous immunosuppressive therapy following organ transplantation.

A surprising finding was that HHV-8 was detected in numerous samples (49% of all samples tested) with a clear difference between HIV-positive and HIV-negative specimens (68% vs 21%). Previous studies detected HHV-8 in NHLs and FHs but to a lesser extent than in this series. Bigoni et al. [1996] detected HHV-8 DNA in 7 of 80 HIV-negative NHLs (9%) and in 1 of 11 HIV-negative reactive lymphadenopathies (9%) but not in 10 AIDS-associated NHLs. In contrast, Otsuki et al. [1996] found HHV-8 in 4 out of 24 AIDS-associated malignant lymphomas (17%). Luppi et al. [1996b] also found HHV-8 DNA in 1 of 33 (3%) AIDS-associated NHLs. Both groups failed to detect the virus in any of the HIV-negative NHLs tested. In order to explain the unexpected high rate of HHV-8 detection in our study, the first point to consider was a possible cross-contamination between samples during DNA preparation or PCR amplification. Several reasons led us to rule out this hypothesis. The strict measures undertaken to avoid intersample carryover proved efficient in other studies [Dupin et al., 1995; Corbellino et al., 1996a]. The negative controls and blank reactions pro-

TABLE II. Viral Load of HHV-8-Positive Samples

Histology	HIV status	No. of HHV-8 DNA copies per cell[a]		Significant differences in two-group comparisons (*P* value)*			
		Median	Range				
PTLD	–	3×10^{-2}	10^{-2}–10^{-1}	x	x		
BL	+	10^{-3}	10^{-4}–10^{-2}	x		x	
LCL	+	10^{-2}	10^{-4}–10^{-1}			x	x
	–	10^{-3}	10^{-4}–10^{-1}				
FH	+	10^{-4}	10^{-4}–10^{-3}		x		x
	–	10^{-3}	10^{-4}–10^{-2}				
All samples	NA	10^{-3}	10^{-4}–10^{-1}				
				0.003	0.002	0.01	0.001

[a]The primary effusion lymphoma case (10^2 copies per cell) was excluded from analysis.
*Only significant differences evidenced from all pairwise comparisons are cited. The groups compared are indicated by crosses.
NA, not applicable.

vided consistently negative PCR results throughout the entire study. Samples were tested blindly at random and contaminations in this case would not have led to the detection of clearly significant differences according to HIV status. Quantitative PCR assays provided concordant results as compared to qualitative detection PCR. A more suitable explanation was that the PCR assay was very sensitive as compared to other studies: we used a DNA input of 1 μg, a 40-cycle program and a readout including hybridization with radioactive probes and 48-hr exposure autoradiography. As an example, preliminary experiments showed that a change from 35 up to 40 cycles induced a gain of sensitivity of about one log (not shown).

The higher frequency of HHV-8 detection in HIV-positive subjects as compared to HIV-negative ones remained intriguing. It could not be explained by the higher sensitivity of the PCR assay and was not in agreement with the results observed in other reports [Bigoni et al., 1996]. In addition, it did not match the quantitative results of our study, which tended to show that HHV-8 load was not influenced by HIV infection. Recent seroepidemiological studies have shown that HHV8 infection is not ubiquitous and is probably transmitted sexually [Gao et al., 1996; Kedes et al., 1996; Simpson et al., 1996]. This infection seems to be confined to subjects with Kaposi's sarcoma or at increased risk of this disease, although the general population may be infected as well, but to a lower extent. Accordingly, HIV-positive hemophiliacs and intravenous drug users are not frequently infected with HHV-8. Therefore, the higher frequency of intratumoral HHV-8 detection for the HIV-positive subjects of our study might simply reflect a higher rate of HHV-8 infection in this group because most of them were homosexual men. Conversely, the lack of intratumoral HHV-8 detection in other studies might be related to the fact that most AIDS patients tested were intravenous drug users [Gaidano et al., 1996]. The higher rate of HHV-8 infection for the HIV-positive patients of our study then reflects common epidemiological risk factors for the two viruses, rather than a putative complex interaction between both in vivo. Accordingly, virus load was not influenced by HIV infection.

Finally, the analysis of intratumoral of HHV-8 load might reconcile the various apparently discrepant facts present within our study and other studies reported. The key finding is that HHV-8 load is very low and close to the threshold of detection assays in most cases. A lack of sensitivity of PCR may then lead to completely negative results. One exception was the primary effusion lymphoma case of our study, which exhibited a virus load of 10^2 copies per cell. This is consistent with HHV-8 loads originally demonstrated in primary effusion lymphomas [Cesarman et al., 1995] and in perfect agreement with the idea that primary effusion lymphoma is a unique biologic entity that should be distinguished from other NHLs [Nador et al., 1996]. As observed, the intratumoral load of lymphoid tissue might be also increased in the case of association with Kaposi's sarcoma. The virus load of 1 copy per cell observed in two Kaposi's sarcoma-associated cases (withdrawn from the final analysis) probably reflected the higher number of infected "passenger" cells the presence in blood has been previously reported [Whitby et al., 1995]. In all the other cases, the virus load ranged from 10^{-4} up to 10^{-1} copy per cell and HHV-8 infection then implicated very few cells in the tumor. These cells are assumed to be either passenger blood cells or latently infected resident ones. Our results tended to show that their number and/or the level of virus reactivation in these cells were increased in PTLDs and HIV-positive LCLs. These lymphoid malignancies corresponded to situations of deeper immunosuppression than HIV-positive BLs and FHs. This suggests a stimulating role of immunodeficiency on virus multiplication, which is in agreement with previous reports [Whitby et al., 1995] and that observed for other herpesviruses. However, in view of the low virus load in tumors excepted for primary effusion lymphomas, it seems unlikely that HHV-8 is a direct causative agent of immunodeficiency-associated B-cell malignancies.

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